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Ameliorating Role of Rutin on Oxidative Stress Induced by Iron Overload in Hepatic Tissue of Rats

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Abstract: Iron is an essential element that participates in several metabolic activities of cells; however, excess iron is a major cause of iron-induced oxidative stress and several human diseases. Natural flavonoids, as rutin, are well-known antioxidants and could be efficient protective agents. Therefore, the present study was undertaken to evaluate the protective influence of rutin supplementation to improve rat antioxidant systems against IOL-induced hepatic oxidative stress. Sixty male albino rats were randomly divided to three equal groups. The first group, the control, the second group, iron overload group, the third group was used as iron overload+rutin group. Rats received six doses of ferric hydroxide polymaltose (100 mg kg^{-1} b.wt.) as one dose every two days, by intraperitoneal injections (IP) and administerated rutin (50 mg kg^{-1} b.wt.) as one daily oral dose until the sacrificed day. Blood samples for serum separation and liver tissue specimens were collected three times, after three, four and five weeks from the onset of the experiment. Serum iron profiles total iron, Total Iron Binding Capacity (TIBC), Unsaturated Iron Binding Capacity (UIBC), transferrin (Tf) and Transferrin Saturation% (TS%)}, ferritin, albumin, total Protein, total cholesterol, triacylglycerols levels and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined. Moreover, total iron in the liver, L-malondialdehyde (L-MDA), glutathione (GSH), Nitric Oxide (NO) and Total Nucleic Acid (TNA) levels and glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activities were also determined. The obtained results revealed that, iron overload (IOL) resulted in significant increase in serum iron, TIBC, Tf, TS% and ferritin levels and AST and ALT activities and also increased liver iron, L-MDA and NO levels. Meanwhile, it decreased serum UIBC, total cholesterol, triacylglycerols, albumin, total protein and liver GSH, TNA levels and Gpx, CAT and SOD activities when compared with the control group. Rutin administration to iron-overloaded rats resulted in significant decrease in serum total iron, TIBC, Tf, TS%, ferritin levels and AST and ALT activities and liver total iron, L-MDA and NO levels with significant increases in serum UIBC, albumin, total protein and total cholesterol levels and in liver GSH, CAT and SOD activities compared with the IOL group. This study provides in vivo evidence that rutin administration can improve the antioxidant defence systems against IOL-induced hepatic oxidative stress in rats. This protective effect in liver of iron-loaded rats may be due to both antioxidant and metal chelation activities.

Key words: Iron overload, oxidative stress, mitochondrial dysfunction, antioxidant enzymes, rutin, rats

INTRODUCTION

Iron is an essential micronutrient for all living organisms and is involved in fundamental enzymatic reactions, such as oxygen metabolism, electron-transfer processes and synthesis of DNA and RNA (Galleano *et al*., 2004). In the human body excessive amounts of iron may become very toxic due to it lacks effective mechanisms to protect cells against iron overload (Siah *et al*., 2005).

Iron overload is one of the most common metal-related toxicity (Zhao *et al*., 2005). It may be caused by: (1) Defects in iron absorption, as increase in iron absorption from the diet as in hereditary hemochromatosis (Beutler, 2007), in which genetic defects alter the regular absorption of iron from the gut or due to iron excess in diet as in Bantu siderosis (Gordeuk *et al*., 2003) (2) Parenteral iron administration in transfusion-dependent anaemias, as β-thalassemia; (3) Pathological conditions characterized by increases in iron (Crisponi and Remelli, 2008).

The liver is the main storage organ for iron, in iron overload, free radical formation and generation of lipid peroxidation (LPO) products, as iron excess generate

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oxidative stress through an increase rate of $HO^{\bullet}$ generation by the Haber-Weiss reaction (Halliwell and Gutteridge, 1984), may result in progressive tissue injury as fibrosis (Arezzini *et al*., 2003) and eventually cirrhosis or hepatocellular carcinoma (Siah *et al*., 2005). In hepatocytes, as excessive iron deposition also lead to further injuries such as hepatocellular necrosis (Olynyk *et al*., 1995). Cases of acute iron toxicity are rare and mostly related to hepatoxicity (Tenenbein, 2001), which in turn cause the oxidation of lipids, proteins, nucleic acids (Papanastasiou *et al*., 2000) that may affect membrane fluidity and permeability and subsequently cell structure, function and viability (Maaroufi *et al*., 2011).

Iron-removal therapy may be achieved by antioxidants, iron chelators and/or free radical scavenging compounds, as flavonoids (polyphenols) (Blache *et al*., 2002), they exert multiple biological effects, including antioxidant and free radical scavenging activities (Negre-Salvayre and Salvayre, 1992), in which the metal chelation can be responsible, partly, both for the antioxidant capacity and some other activities (e.g., inhibition of lipooxygenases by chelation/reduction of iron in their active sites) (Vasquez-Martinez *et al*., 2007). Rutin is a kind of flavonoid glycoside known as vitamin P, a polyphenolic compound that is widely distributed in vegetables and fruits (Hertog *et al*., 1993), it is very effective free radical inhibitor in animal and human pathological states, such as IOL in rats (Afanas'ev *et al*., 1995), including hepatoprotective (Janbaz *et al*., 2002), in which its administration sharply suppressed free radical production in liver microsomes and by phagocytes in IOL animals (Afanas'ev *et al*., 1995) and also could reduce iron content in mouse liver (Gao *et al*., 2002). Accordingly, the aim of the present study was to evaluate the protective role of rutin administration against hepatic oxidative stress induced in iron-loaded rat models.

MATERIALS AND METHODS

Experimental animals: Sixty white male albino rats of 8-10 weeks old and weighing 180-220 g were used in the experimental investigation of this study. Rats were obtained from the Laboratory Animals Research Center, Faculty of Veterinary Medicine, Benha University, housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of the experiment. The animals provided with a constant supply of standard pellet diet and fresh, clean drinking water ad-libitum.

Drug and antioxidants: The drug and antioxidant compounds used in the present study were:

- **Haemojet(R):** Haemojet ampoules were produced by Amriya Pharm. Ind. for European Egyptian Pharma. Ind., Alexandria, Egypt. Haemojet was obtained as pack of three ampoules of two ml solution. Each ampoule contains elemental iron (100 mg) as ferric hydroxide polymaltose complex
- **Rutin:** Rutin is pale yellow crystalline powder (purity~99%). It was purchased from EIPICO 'Egyption Pharmaceutical Industries Company, 10th of Ramadan city, Egypt. Rutin was dissolved in propylene glycol and administered to animals in daily oral dose of 50 mg kg^{-1} (b.wt.) (Fernandes *et al*., 2010)

Induction of iron overload: Iron overload was induced by intraperitoneal injections of six doses (three doses per week) of ferric hydroxide polymaltose complex of 100 mg kg^{-1} b.w. (Zhao *et al*., 2005).

Experimental design: Rats were randomly divided into three equal groups: Each group containe twenty rats as follows:

Group I: (control group): Received saline only and served as control for all other groups

Group II: (iron overload): Received six doses (three doses per week) of 100 mg kg^{-1} b.wt. of ferric hydroxide polymaltose administerated as IP-injections

Group III: (iron overload+rutin): Received six doses (three doses per week) of 100 mg kg^{-1} b.wt. of ferric hydroxide polymaltose by IP-injections, followed by daily oral administration of rutin at a dose level of 50 mg kg^{-1}b.wt. until the sacrificed day

Sampling: Random blood samples and liver tissue specimens were collected from all animals groups (control and experimental groups) three times along the duration of experiment at three, four and five weeks from the onset of rutin administration.

Blood samples: Blood samples were collected by ocular vein puncture in dry, clean and screw capped tubes and serum was separated by centrifugation at 2500 rpm for 15 min. The clean, clear serum was separated and received in dry sterile samples tube, then kept in a deep freeze at -20°C until used for subsequent biochemical analysis

Liver tissues: At the end of the each experimental period, rats were sacrificed by cervical decapitation. The liver specimen was quickly removed and weighted, then

perfused with cold saline to exclude the blood cells and then blotted on filter study and stored at -20°C. Briefly, half of liver tissues were cut, weighed and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates. The homogenates were centrifuged at 5,000 rpm for 15 min at 4°C then the supernatant was used for the subsequent biochemical analysis.

The other half of livers were weighed and putted into glass flask, then 5 volumes of mixed acid (nitric acid: Perchloric acid, 4:1) were added, heated until large amount of white vapors could be seen. The volumes of the digested samples were adjusted to 10 mL with double distilled water, then the obtained solutions were used to analyze iron contents.

Biochemical analysis: Serum iron and total iron binding capacity (TIBC), ferritin, albumin, total protein, total cholesterol, triacylglycerols levels and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined according to the methods described by Makino (1988), Dawson *et al.* (1992), Young (2001), Gendler (1984), Goodman *et al.* (1988), Stein (1987) and Young (2001), respectively.

Moreover, total iron in the liver, L-malondialdehyde (L-MDA), reduced glutathione (GSH), Nitric Oxide (NO) and Total Nucleic Acid (TNA) levels and Glutathione Peroxidase (GPx), catalase (CAT) and Superoxide Dismutase (SOD) activities were determined according to the methods described by Parker *et al.* (1967), Esterbauer *et al.* (1982), Beutler (1957), Montgomery and Dymock (1961), Spirin (1958), Gross *et al.* (1967), Sinha (1972) and Packer and Glazer (1990), respectively.

Statistical analysis: The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple test. All analyses were performed using the statistical package for social science 13.0 software, (SPSS, 2009). Values of $p<0.05$ were considered to be significant.

RESULT

The results presented in Table 1 and 2 revealed that, iron overload resulted in significant increase in serum iron, TIBC, Tf, TS% and ferritin levels and AST and ALT activities and also increased liver iron, L-MDA and NO levels. Meanwhile, it decreased serum UIBC, total cholesterol, triacylglycerols, total protein and albumin and liver GSH and TNA levels and Gpx, CAT and SOD activities, compared with the control group. Rutin administration to iron-loaded rats resulted in significant decreased in serum iron, TIBC, Tf, TS%, ferritin levels, AST and ALT activities and liver iron, L-MDA and NO levels, with significant increases in serum UIBC, total protein, albumin and total cholesterol levels and in liver GSH level, CAT and SOD activities, compared with the IOL group.

DISCUSSION

Iron overload in rats is an excellent model to study the *in vivo* LPO in which excess iron induced oxidative stress by increasing lipid peroxide levels in liver and in serum (Reddy and Lokesh, 1996). Subsequently, MDA and 8-IP adducts that were formed, significantly contributed to liver damage that is assessed by AST and ALT levels in the iron-supplemented rats (Asare *et al.*, 2006). Excess hepatic iron may thus cause peroxidation of membrane lipids and oxidative liver injury (Abel and Gelderblom, 1998) in which IOL enhances liver injury and accelerates the process of fibrosis (Arezzini *et al.*, 2003). This tissue injury can be relieved by the administration of an appropriate chelating agent which can combine with the iron and increase its rate of excretion (Zhao *et al.*, 2005), as flavonoids that are substances with both chelating and free radical scavenging properties (Fraga and Oteiza, 2002) thus, rutin may be a very useful medicine for treatment (Reddy and Lokesh, 1996).

Serum and liver iron and serum TIBC, Tf, TS% and ferritin levels were significantly elevated in the iron-loaded rats, while serum UIBC was significantly decreased (Table 1, 2). These results came in accordance with the data of Silva *et al.* (2008) that reported, serum iron and TS% was 75% higher in rats with iron-dextran treatment when compared with the untreated control group. Also, Junge *et al.* (2001) reported that, acute iron overload elicited significant enhancement in iron levels of rats livers and Nahdi *et al.* (2010) observed that, IOL elicited significant enhancement in serum iron and significant increase (>10-fold) in liver iron in rats, TS was more than 100%, that most certainly results in the presence of Non Transferrin Binding Iron (NTBI) catalyzing the formation of reactive radicals (Zhang *et al.*, 2006). Moreover, Crisponi and Remelli (2008) found that, when the iron load increases, the Iron Binding Capacity (IBC) of serum Tf is exceeded and a NTBI fraction of plasma iron appears which generates free hydroxyl radicals and induces dangerous tissue damage. Additionaly, Theurl *et al.* (2005) reported that, liver ferritin levels were increased with prolonged iron challenge as iron initially accumulated in spleen macrophages with subsequent increase in macrophage ferroportin and ferritin expression.

Table 1: Effect of treatment with rutin on some biochemical blood parameters in iron overloaded male rats

	Animal groups								
	Control (weeks)			Iron overload (weeks)			Rutin+iron overload (weeks)		
Parameters	3	4	5	3	4	5	3	4	5
Iron (μg dL^{-1})	138.50±7.03^{b}	144.50±3.66^{c}	161.00±7.82^{b}	230.75±3.52^{a}	232.80±8.22^{a}	260.75±11.84^{a}	158.00±8.96^{b}	176.80±5.28^{b}	185.50±4.52^{b}
TIBC (μg dL^{-1})	211.50±5.30^{c}	221.25±4.46^{d}	243.50±10.99^{b}	289.25±2.75^{a}	300.00±7.34^{a}	336.50±14.86^{a}	239.75±4.17^{b}	272.00±6.92^{b}	271.50±4.29^{b}
UIBC (μg dL^{-1})	75.00±2.27^{a}	76.75±0.95^{b}	82.00±3.44^{ab}	54.75±0.85^{b}	58.75±2.81^{c}	73.75±2.25^{b}	81.75±7.43^{a}	96.00±0.71^{a}	86.25±2.10^{a}
Transferrin (g L^{-1})	1.50±0.04^{c}	1.55±0.03^{a}	1.70±0.08^{b}	2.04±0.04^{a}	2.10±0.04^{a}	2.37±0.10^{a}	1.70±0.04^{b}	1.90±0.02^{a}	1.91±0.02^{b}
TS (%)	65.25±1.70^{bc}	65.25±0.25^{b}	66.50±0.65^{b}	79.75±1.32^{a}	79.25±1.25^{a}	77.50±0.29^{a}	63.25±5.44^{c}	64.75±0.85^{b}	69.50±1.50^{b}
Ferritin (ng mL^{-1})	2.07±0.08^{b}	2.25±0.03^{b}	3.33±0.04^{b}	2.47±0.05^{a}	2.60±0.04^{a}	3.75±0.07^{a}	2.35±0.03^{a}	2.45±0.03^{a}	3.33±0.04^{b}
AST (U L^{-1})	78.60±6.07^{d}	94.00±1.58^{c}	72.40±6.17^{c}	152.60±5.56^{a}	149.80±4.93^{a}	148.00±6.63^{a}	112.60±7.69^{c}	117.80±5.18^{b}	132.00±1.41^{b}
ALT (U L^{-1})	14.80±1.28^{c}	15.60±0.75^{c}	16.60±1.21^{b}	25.60±1.08^{a}	34.40±2.11^{a}	31.80±2.35^{a}	19.60±1.54^{b}	22.80±1.88^{b}	19.20±1.16^{b}
Cholesterol (mg dL^{-1})	110.25±5.65^{a}	90.76±7.44^{ab}	89.25±7.98^{b}	68.75±9.43^{b}	78.75±4.23^{b}	77.00±2.38^{b}	114.00±2.12^{a}	103.76±2.32^{a}	109.00±7.36^{a}
Triacylglycerols (mg dL^{-1})	94.00±5.80^{a}	78.00±3.87^{a}	82.00±5.67^{a}	74.50±4.17^{b}	66.75±4.77^{a}	71.75±1.32^{a}	92.25±5.60^{a}	78.50±2.40^{a}	81.25±2.46^{a}
Albumin (g dL^{-1})	3.02±0.17^{b}	3.44±0.18^{a}	3.84±0.39^{a}	2.04±0.13^{c}	2.72±0.08^{b}	1.98±0.10^{b}	3.98±0.32^{a}	3.88±0.19^{a}	4.40±0.20^{a}
Total protein (g dL^{-1})	6.36±0.31^{b}	6.90±0.44^{ab}	7.00±0.64^{a}	4.58±0.25^{c}	5.92±0.25^{b}	3.44±0.22^{b}	6.90±0.25^{b}	7.62±0.50^{a}	7.02±0.37^{a}

Data are presented as (Mean±SE), SE: Standard error, Mean values with different superscript letters in the same row are significantly different at ($p<0.05$)

Thus, iron overload treatment suggesting a novel mechanistic link between dopaminergic GSH depletion and increased Fe levels based on increased translational regulation of transferrin receptor 1(TfR1) (Kaur *et al*., 2009), in which iron deposition and related damages in liver indicate a strong relation between alterations of cellular redox condtion/increase in ROS generation due to GSH depletion with altered iron homeostasis in hepatic cell that led to iron deposition (Tapryal *et al*., 2010). However, exess iron induced increase in hepcidin mRNA level that was not sufficient to prevent increased intestinal iron absorption and onset of IOL compatible with the observation that serum iron was very high in that condition and TS was more than 100% that most certainly resulted in the presence of NTBI (Nahdi *et al*., 2010). Also, ferritin induction is expected to occur under conditions of chronic IOL when intracellular iron is abundant, due to lack of binding of the iron regulatory protein1 (IRP-1) to Iron-Responsive Elements (IRE) and IRP-2 degradation, resulting in significant translation of ferritin mRNA (Kim and Ponka, 2003). In cases of iron overload, the natural storage and transport proteins such as ferritin and transferrin become saturated and overwhelmed and then the iron spills over into other tissues and organs; At the same time, oxidative stress arises because of the catalytic activity of the metal ion on producing high reactive oxygen radicals and finally leads to tissue injury (Zhao *et al*., 2005).

When rats were administerated with rutin serum and liver iron and serum TIBC, Tf, TS% and ferritin concentrations were significantly decreased and serum UIBC was significantly increased than that of iron-loaded group (Table 1, 2). Similarly, Gao *et al*. (1999) found that, iron contents were significantly decreased in the liver of rutin and baicalin fed rat.

Also, Gao *et al*. (2002) reported that, oral adminstration of higher doses of rutin in mice can cause a decrease of serum iron, copper and zinc concentrations. In addition to Gao *et al*. (2003) that reported, the iron contents, in the liver of rutin or baicalin containing diet (1%) fed rats were significantly decreased. Moreover, Zhao *et al*. (2005) observed that, when iron-loaded mice were supplemented with baicalin, there was a decrease of hepatic total iron, at the same time, serum nonheme iron was significantly increased, indicating that baicalin could gradually combine with hepatic non-heme iron and finally excreted it from the body. Moreover, Zhang *et al*. (2006) found that, the increased NTBI in quercetin supplemented mice caused no further oxidation indicating that the increased serum non-heme iron may come from flavonoids chelated Fe and although serum ferritin level was still higher than that of normal mouse, it was significantly decreased compared with IOL-mouse. Furthermore, Kostyuk *et al*. (2004) observed that, quercetin effectively released Fe from ferritin and Fe-flavonoid complexes are demonstrated to be antioxidants. These results can be attributed to metal chelating effects of rutin, which are involved in the Fenton reaction (Arjumand *et al*., 2011) that can be responsible for the documented antioxidant capacity of flavonoids (Mladenka *et al*., 2011), as these chelation effects of flavonoids are structure specific (Gao *et al*., 2003) and suggests that the high reducing power and metal chelating activities mechanisms may play a key role in the inhibition of oxidative processes

Table 2: Effect of treatment with rutin on some biochemical liver parameters in iron overloaded male rats

	Animal groups								
	Control (weeks)			Iron overload (weeks)			Rutin+iron overload (weeks)		
Parameters	3	4	5	3	4	5	3	4	5
Total iron (μg dL^{-1})	290.00±17.32^{c}	323.33±14.53^{c}	353.33±26.03^{c}	1266.67±46.67^{a}	1313.33±17.64^{a}	1373.33±12.02^{a}	1140.00±45.83^{ab}	1130.00±35.12^{b}	1204.33±83.96^{ab}
MDA (nmol g^{-1})	131.60±5.07^{b}	139.80±6.99^{b}	136.00±8.33^{c}	189.60±18.38^{a}	197.80±9.34^{a}	182.00±5.72^{a}	128.80±7.18^{b}	132.40±11.02^{b}	154.40±9.20^{bc}
GSH (nmol g^{-1})	87.60±3.75^{a}	82.60±1.50^{a}	80.00±0.89^{a}	76.00±1.23^{b}	75.40±0.93^{b}	73.60±0.93^{b}	79.00±0.71^{b}	78.60±0.93^{b}	77.80±1.24^{a}
GP_X (U g^{-1})	0.49±0.02^{b}	0.54±0.01^{a}	0.17±0.02^{a}	0.58±0.01^{a}	0.44±0.02^{bc}	0.23±0.02^{a}	0.56±0.02^{a}	0.48±0.01^{b}	0.18±0.02^{a}
CAT (U g^{-1})	14.78±0.33^{a}	14.94±0.23^{a}	14.84±0.29^{a}	12.16±0.37^{b}	12.46±0.39^{b}	12.72±0.29^{b}	15.50±0.13^{a}	14.20±0.18^{a}	14.22±0.20^{a}
SOD (U g^{-1})	304.00±19.65^{a}	270.00±7.07^{a}	278.00±18.55^{a}	174.40±10.27^{c}	174.00±16.31^{b}	145.00±9.22^{c}	270.00±25.50^{b}	260.00±10.49^{a}	230.00±14.83^{b}
NO (μmol g^{-1})	0.159±0.002^{b}	0.164±0.002^{b}	0.153±0.003^{b}	0.193±0.004^{a}	0.225±0.02^{a}	0.176±0.004^{a}	0.154±0.002^{b}	0.159±0.002^{b}	0.131±0.001^{d}
TNA (μg g^{-1})	0.43±0.04^{a}	0.37±0.03^{a}	0.45±0.04^{a}	0.32±0.04^{a}	0.25±0.03^{b}	0.33±0.03^{a}	0.37±0.06^{a}	0.36±0.03^{ab}	0.43±0.04^{a}

Data are presented as (Mean±SE), SE: Standard error, Mean values with different superscript letters in the same row are significantly different at ($p<0.05$)

(Lue *et al*., 2010). However, although rutin form chelates with Fe ions, it is hydrolyzed by the intestinal flora to its corresponding aglycone, quercetin (Prince and Priya, 2010), which is responsible for its in vivo antioxidant activity, therefore, radical scavenging activity of rutin may be more important than their metal chelating activity (Kim *et al*., 2011).

Iron overload resulted in significant increase in serum AST and ALT activities compared with the normal control rats. The obtained results are nearly similar to data reported by Asare *et al*. (2006) that showed, in the iron-supplemented rats all of the indices of LPO, including AST and ALT, were increased significantly (~5-fold) compared with the control. Also, Silva *et al*. (2008) observed that, administration of iron dextran to rats increased AST activity, a marker for mitochondrial lesions and had no effect on ALT. As AST and ALT were used as sensitive indicators of liver damage (Mahmoud, 2012), the serum enzymes increased activities in iron-loaded rats can be attributed to the generation of ROS and oxidative damage by excess hepatic iron that may result in chronic necroinflammatory hepatic disease, which in turn generates more ROS and causes additional oxidative damage (Jungst *et al*., 2004). Rutin administration in iron-loaded rats decreased serum transaminases activities (Table 1). Similar results were reported by the data of Fernandes *et al*. (2010) that recorded, rutin administration to streptozotocin-diabetic rats decreased serum ALT and AST activities compared to untreated controls. Also, Mahmoud (2012) observed that, rutin administration significantly decreased the levels of AST and ALT activities in hyperammonemic rats, suggesting protection by preserving the structural integrity of the hepatocellular membrane against ammonium chloride. Rutin also scavenged free radicals (Cillard *et al*., 1990) and inhibiting LPO process (Karthick and Prince, 2006), in which the iron-rutin complexe studied not only retained the antioxidant properties of rutin, but in many cases exhibited enhanced free radical-scavenging activity (Ostrakhovitch and Afanas'ev, 2001).

The obtained results revealed that, iron overload resulted in significant decrease in serum total protein and albumin levels. The results agree well with those recorded by Asare *et al*. (2006) that found, iron accumulation disrupts the cell redox balance and geneates chronic oxidative stress, which damages DNA, lipids and protein in hepatocytes leading to both necrosis and apoptosis. That can be explained by protein oxidation that is known to give rise to alterations in both the backbone and side chains of the molecule, leading to the denaturation and loss of biological activities of various important proteins and cell death (Zhang *et al*., 2006), in which ROS may also cause oxidative damage to polyunsaturated fatty acids of membrane phospholipids, LPO enhanced by high-iron diet (Lafay *et al*., 2005), releasing cytotoxic and reactive aldehyde metabolites as MDA (Asare *et al*., 2006). Those cytotoxic products may impair cellular functions, including nucleotide and protein synthesis (Cheeseman, 1993). This suggestion was confirmed also by Youdim *et al*. (2005) that reported, ROS are capable of oxidizing cellular proteins, nucleic acids and lipids.

Rutin administration to iron-overloaded rats induced significant increases in serum total protein and albumin concentrations compared with the IOL-group. Similar results were reported by Zhang *et al*. (2006) that recorded ring the treatment with baicalin and quercetin in IOL-induced mice liver injury, the reduction of liver protein oxidation can be considered as a sign of protection under IOL. Kamalakkannan and Prince (2006) also observed that, oral administration of rutin to diabetic rats lead to significant increase in the plasma total protein and albumin concentrations when compared with the diabetic control. The antioxidant activity of rutin in Fenton reaction (Caillet *et al*., 2007) may explain the reduction of protein oxidation, as a mechanism of action preventing protein oxidation, in which the inhibitive effects of flavonoids on protein oxidation may come from the combination of both iron eliminating and direct free radical scavenging activities (Zhang *et al*., 2006).

Serum total cholesterol and triacyglycerols levels were significantly decreased in the iron loaded rats. These obtained results may be explained by Silva *et al*. (2008) that reported, hepatic injury triggered by iron excess may increase the concentration of secondary serum metabolites, such as cholesterol, triacylglycerols and glucose and also recorded that, treatment with Fe-dextran in male rats increased serum triacylglycerols level, but had no effect on the cholesterol evel. However, Turbino-Ribeiro *et al*. (2003) reported that, absence of alteration in serum cholesterol in rabbits receiving Fe-dextran injections.

When rats were administerated with rutin, serum total cholesterol and triacyglycerols levels were significantly increased than that of iron loaded control group. Contradictory results were obtained by Fernandes *et al*. (2010) reported that, serum total cholesterol and Low Density Lipoprotein (LDL)-cholesterol levels were lowered in the rutin-treated diabetics rats group. Also, Park *et al*. (2002) recorded that, supplementation of 0.1% rutin and tannic acid significantly lowered both plasma total cholesterol and triacylglycerols compared with control. These results may be explained by the lose of the amphiphilic properties of rutin, that be less capable of scavenging free radicals from the most lipophilic regions

of the LDL particle (cholesterol esters and triacylglycerols) (Lue *et al*., 2010). In which the decrease in cholesterol level was due exclusively to the LDL and VLDL fraction (Aggarwal and Harikumar, 2009). On the other hand, Jiang *et al*. (2007) showed a dose-response effect of rutin in inhibiting LDL peroxidation and the reduced CAT activity in the rutin and tannic acid treatment might lead to less cholesteryl ester being available for VLDL packing, thereby resulting in a reduction in its secretion from the liver (Carr *et al*., 1992).

Liver L-MDA concentrations were significantly elevated in the iron-loaded rats as compared with control group. The obtained results are nearly similar to those of Kokoszko *et al*. (2008) who obseved that, the injection of FeCl or $FeSO_4$ (3 mg Fe^{2+}/100 g b.w.) significantly increased LPO products in the rat liver. Also, Nahdi *et al*. (2010) reported that, IOL in rats was accompanied by enhancement in LPO as shown by a significant increase in all tissue MDA concentration in iron supplemented group except the spleen and added that, in the liver there was a perfect correlation between MDA level and tissue iron content, suggesting production of oxidative stress. LPO generated by ROS was measured in terms of MDA (Arjumand *et al*., 2011), that is a secondary end product of the oxidation of polyunsaturated fatty acids (Yang *et al*., 2008). Focusing on the liver organ, the cytotoxic degradation products as MDA and 4-hydroxy-2-non-enal (4-HNE) (Esterbauer *et al*., 1991) can form covalent adducts with proteins, phospholipids and DNA (Guichardant *et al*., 1998), in which the formation of liver microsomal MDA protein adducts, during IOL in mice and the microsomal function impairment may alter protein function and might lead to cellular injury and Fe-associated hepatotoxicity (Valerio and Petersen, 1998). When rats were administerd with rutin hepatic L-MDA concentrations were significantly decreased than that of iron-loaded control group (Table 2). The obtained results agree with the data of Ostrakhovitch *et al*. (1995) that reported, rutin administration in IOL-rats sharply decreased microsomal LPO in the liver and spontaneous oxygen radical production by peritoneal macrophages. Also, Gao *et al*. (2003) reported that, LPO level in the liver of the rutin fed group was significantly decreased in comparison to the control group, As iron overload led to enhancement in LPO (Nahdi *et al*., 2010), some flavonoids can be both antioxidants and iron chelators; it means that flavonoids will be good candidates for curing IOL related diseases, as they can play a double role in reducing the rate of oxidation, one act as iron chelator (Borsari *et al*., 2001) and the other act as radical trap (Van Acker *et al*., 1998). Afanas'ev *et al*. (1995) also found that, rutin administration sharply suppressed free radical production in liver microsomes by phagocytes in iron overload rats. This antioxidant activity due to the inactive iron-rutin complexes of rutin that may be a good in vivo antioxidant and may be more effective free radical scavengers compared to the parent rutin (Ostrakhovitch and Afanas'ev, 2001). The sharp decrease in L-MDA in IOL-rats may be also attributed to the free radical scavenging property of rutin, in which IOL in rats induces the oxidative stress that was characterized by oxygen radical overproduction in liver microsomes, peritoneal macrophages and blood neutronphils (Mahmoud, 2012).

The obtained results revealed that, IOL resulted in significant decrease in liver GSH levels when compared with the control group. The obtained data are nearly similar to those reported by Poli *et al*. (2004) that observed, the increase in protein carbonylation and reduction in GSH content as well as in the GSH/GSSG ratio of the liver were observed after 6 weeks of treatment probably induced by iron-generated free radical activity (Pietrangelo, 2003). Also, Jagetia and Reddy (2011) reported that, introduction of Fe into mouse liver mitochondrial fraction caused a time dependent depletion in GSH (~2-fold) lower than control at 30 min post treatment. GSH is a major non-enzymatic tripeptide multifunctional intracellular antioxidant (Morimoto *et al*., 2008) that was often used as an estimation of the redox environment of the cell (Schafer and Buettner, 2001). Iron deposition and related damages in liver indicate a strong relation between alterations of cellular redox condtion/increase in ROS, e.g., Fe-induced free radical (Pietrangelo, 2003) due to GSH depletion, suggesting a novel mechanistic link between dopaminergic GSH depletion and increased iron levels (Kaur *et al*., 2009); In conclusion, reduced GSH functions intracellularly to reduce numerous oxidizing compounds, including ROS (Gong *et al*., 2010).

Rutin administration to iron-overloaded rats resulted in significant increase in liver GSH level compared with the IOL-group. Similarly, Korkmaz and Kolankaya (2010) observed that, rutin pretreatment significantly protected against the severe depletion of GSH content and MnSOD activity in the I/R-induced damage in rat kidney. Moreover, Arjumand *et al*. (2011) reported that, rutin treatment in cisplatin administered rats showed significant improvement in GSH concentration, suggesting its role in scavenging the free radicals generated by cisplatin-induced renal inflammation and apoptosis. The antioxidant imbalance was compensated by the prophylactic treatment of rutin as excessive LPO can cause increased GSH consumption (La Casa *et al*., 2000), this inhibitory effects of rutin on in vivo free radical

production in IOL-rats are probably explained by its ability to form inactive iron-rutin complexes (Afanas'ev *et al*., 1989).

The obtained results revealed that, iron overload resulted in significant decrease in liver Gpx, CAT and SOD activities when compared with the control group. Iron overload can destruct the balance between prooxidants and antioxidants, leading to severe loss of total antioxidant status level. This phenomenon can be seen in most iron overload animal models (Dabbagh *et al*., 1994) as well as iron overload disease such as hereditary haemochromatosis (Young *et al*., 1994) and thalassemia (Livrea *et al*., 1996).

Catalase is an iron-containing antioxidant enzyme, it was reported that under iron overload, there was a significant decrease of catalase activity in rat liver (Galleano and Puntarulo, 1997). Likewise, Zhao *et al*. (2005) who showed that, Fe-dextran injection in mouse caused a significant decrease in hepatic CAT activity. Moreover, Valko *et al*. (2006) reported that, GPx significantly competes with CAT for H_2O_2 substrate and it is the major source of protection against low levels of oxidative stress-induced cancer. These results can be explained as the ROS generated during normal cellular processes are immediately detoxified by endogenous antioxidants like GSH, CAT, GPx, glutathione reductase, glutathione-S-transferase, etc. (Kim *et al*., 2006).

SOD work in conjunction with CAT and GP_X (Michiels *et al*., 1994), preventing its interaction with Fe and therefore formation of the highly toxic $^{\bullet}OH$, in which SOD and GP_X are supportive enzyme system of the first line cellular defense against oxidative injury (Kalpravidh *et al*., 2010). GPx decomposes peroxides, that initiate a chain of free radical formation (Jagetia and Reddy, 2011) to H_2O (or alcohol) while simultaneously oxidizing GSH; Significantly, GPx competes with CAT for H_2O_2 substrate, as it is the major source of protection against low levels of oxidative stress (Valko *et al*., 2006) and the increase of SOD/GPx ratio in Fe treated cells compared to control cells indicated that Fe-induced oxidative injury appeared, that might be indicative of ROS increase due to unefficient scavenging by enzymes (Formigari *et al*., 2007).

Rutin administration to iron-loaded rats resulted in significant increases in liver CAT and SOD activities compared with the IOL group (Table 2). As natural antioxidants, flavonoids intake may increase total antioxidant status level in living body, supplementation of baicalin or another flavonoid, rutin, could increase hepatic total antioxidant status level in rats and mice (Gao *et al*., 2003; Zhao *et al*., 2005). This effect may come from the chelation of free iron ion with stopping iron-catalyzed oxidative reaction and the direct increasing antioxidant status by baicalin and its metabolites that acte as strong antioxidant. Thus, the baicalin supplementation introduced a new source of antioxidant and could partly inhibit peroxidation-induced heme destruction and then provided a protection on catalase (Zhao *et al*., 2005). The significant increases in liver antioxidant enzymes in rutin treated iron-loaded rats are in conformity with the data reported by Park *et al*. (2002) that recorded, dietary rutin and tannic acid have a significant effect on SOD and GPx in rats. As a concomitant increase in CAT and/or GPx activity is essential if a beneficial effect from the high SOD be expected. Mahmoud (2012) found that, hyper-ammonemic rats pretreated with rutin significantly increased liver CAT and GPx. Chronic iron administration induced adaptive responses involving stimulation of the antioxidant defenses. Rutin significantly inhibited LPO in IOL-microsomes (Afanas'ev *et al*., 1995) that may be due to the free radical scavenging property (Mahmoud, 2012). The three enzymes can prevent damage by detoxifying ROS. The significant elevation of SOD activity also suggests that its free-radical scavenging activity is only effective when it is accompanied by an increase in activity of CAT and/or GPx activity, because SOD generates H_2O_2 as a metabolite (Park *et al*., 2002), which is more toxic than oxygen radicals in cells and needs to be scavenged by CAT or GPx (Pigeolet *et al*., 1990).

Iron overload resulted in significant increase in liver NO levels when compared with the control group. Similar results were recorded by Cornejo *et al*. (2001) that found, increased NO generation was evidenced in the liver under conditions of acute IOL. Moreover, Vadrot *et al*. (2006) explored that, increased production of NO by nitric oxide synthase-2 was demonstrated in patients with hepatic cell carcinoma complicating hereditary haemochromatosis. That can be explained as NO is an inorganic reactive nitrogen species synthesized in liver by inducible nitric oxide synthase (iNOS) found in hepatocytes, Kupffer cells and endothelial cells (Alderton *et al*., 2001), whose expression is controlled by the redox-sensitive transcription factor, nuclear factor-kappa B (Kleinert *et al*., 2004) and the complex interrelationships between Fe and NO (Galleano *et al*., 2004) can result in changes in in vivo NO production (Kagan *et al*., 2001). Also the increase in rat liver NOS activity due to chronic iron overload (Cornejo *et al*., 2007) is related to upregulation of iNOS expression (Cornejo *et al*., 2005). Rutin administration to iron-overloaded rats resulted in significant decreased liver NO levels compared with the IOL group. The data obtained are in harmony with Chen *et al*. (2001a, b) and Shen *et al*. (2002) that showed, rutin inhibit lipopolysaccharide-induced NO production. NO was

proposed to act as a pro-oxidant at high conc. (Morand *et al*., 2000), or when it reacts with $O_2^{\bullet-}$, forming the highly reactive peroxynitrite (Radi *et al*., 2001) that is suppressed by flavonoids by direct scavenging (Haenen *et al*., 1997).

Iron overload resulted in significant decreased in liver TNA levels when compared with the control group. Similarly, Youdim *et al*. (2005) explored that, iron is a major generator of ROS that lead to damage of lipids, proteins carbohydrates and nucleic acids. Park and Park (2011) reported that, Ferric-nitrilotriacetate markedly induced DNA damage in human leukocytes in vitro and rat leukocytes *in vivo*. The decrease in total nuclic acid level can be attributed to the primary generation of H_2O_2 and $^{\bullet}OH$, due to Fe-induced oxidative stress, that damages DNA and other biomolecules (Huang, 2003). As $^{\bullet}OH$ may damage the nucleotide bases themselves, resulting in oxidized base products such as 8 oxo-guanine and fragmented or ring-opened derivatives (Lu *et al*., 2001). Iron also is thought to be involved in β-cleavage of lipid hydroperoxides, producing biogenic aldehydes that interact with DNA to form exocyclic products (Kew, 2009) that trigger free radical-mediated chain reaction including LPO, DNA damage and protein oxidation (Gutteridge and Halliwell, 2000).

Rutin administration to iron-overloaded rats, non significantly increased liver TNA concentration compared with the IOL-group. The obtained results are of the same harmony with the data of Undeger *et al*. (2004) that reported, rutin can prevent damage to DNA as it may modulate the enzymes necessary for activation of carcinogens. Also, Gong *et al*. (2010) observed that, rutin (50 μM) blocked H_2O_2-induced apoptosis in human umbilical vein endothelial cells and thus protecting DNA damage. Moreover, Omololu *et al*. (2011) reported that, rutin protect the stability of the genome. This protection against DNA damage is particularly important since oxidative damage to DNA, especially strand breaks, is highly dependent on the amount of Fe bound to DNA (Diaz-Castro *et al*., 2010), in which polyphenols, due to their ability to coordinate iron, are one large class of antioxidants that has been extensively examined for treatment and prevention of conditions associated with iron-generated ROS and oxidative stress (Perron and Brumaghim, 2009), as iron chelation was responsible for prevention of nuclear DNA damage by quercetin (Sestili *et al*., 1998).

CONCLUSION

From the obtained results it could be concluded that, the natural flavonoids, rutin inhibited the adverse effect of ferric ion induced oxidative stress by reducing protein and DNA oxidation and inhibition of lipid peroxidation in liver tissue due to its marked hepatoprotective role in the experimental rats. Rutin treatment improved the cytoprotective enzymatic and non-enzymatic antioxidants as revealead by elevated the GSH concentration, GPx, CAT and SOD activities and thus, might protect the cellular environments from iron-induced free radical damage. The results indicate that, rutin may have potential effects in inhibiting the iron-induced oxidative stress in human. The protective effect of rutin on livers of iron-overloaded rats may be due to its high antioxidant activity, including both its radical scavenging and iron chelation activities. So we recommended using rutin-enriched food regularly with additional research work for medicine manufacturing for protection against the bad complications of IOL-induced oxidative stress.

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